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Aminopeptidase-Like Activity in Hemolymph Plasma from Larvae of the Gypsy Moth, *Lymantria dispar* (Lepidoptera: Lymantriidae)

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ABSTRACT. Aminopeptidase was isolated from the plasma fraction of hemolymph from last instar larvae of the gypsy moth *Lymantria dispar*. Activity was detected using the synthetic substrate L-alanine-4-nitroanilide. Total aminopeptidase activity per microliter of plasma varied with developmental stage. Activity was detected throughout the last (fifth) larval instar and increased throughout the pupal stage. The relatively large volumes of hemolymph that could be collected from day-1, fifth instar larvae made this the stage of choice for plasma preparation and enzyme purification. Hemolymph plasma was fractionated by TSK-DEAE-5PW ion exchange chromatography. Seventy percent of all aminopeptidase-like activity was detected in column fractions eluting near 125 mM NaCl. The enzyme was further purified using a combination of ion exchange and size exclusion (TSK-G2000SW$_{XL}$) methods. Purity was assessed by SDS-polyacrylamide gel electrophoresis. Native and denaturing electrophoresis results suggest that the enzyme, termed AP-125, is a hexamer of approximately 420,000 MW. The enzyme is inhibited competitively by amastatin (2 μM) and noncompetitively by leuhistin (1 μM). AP-125 is a new insect aminopeptidase, differing from those reported thus far in molecular weight, subunit composition and inhibitor response. *Copyright © 1997 Elsevier Science Inc.* comp biochem physiol 116B;1:11–18, 1997.

KEY WORDS. Aminopeptidase, column chromatography, gel electrophoresis, hemolymph, insect development, *Lymantria dispar*, plasma, protease inhibitors

INTRODUCTION

Regulation of the levels of biologically active intercellular proteins is an essential component of normal metabolic activity in animals. Termination of peptide signals is effected primarily by cell-surface proteases (20) and circulating aminopeptidases (19). Cell membrane-associated proteases have been reported in insects (6,7,12,16). The first step in the degradation of circulating peptides in invertebrates involves the cleavage of peptide bonds between internal amino acid residues, through the action of a metalloendopeptidase (4,6,9,10,12,16). However, the rapid clearance of *C*-terminal products of this internal cleavage, and the catabolism of any peptide with an unblocked *N*-terminus, appear to be due to the action of aggressive aminopeptidases (9,16,18). Aminopeptidases were some of the earliest proteases discovered. These enzymes serve, among other functions, in the degradation of circulating hormonal peptides (19). Aminopeptidases have been isolated from insect molting fluid where they are involved with cuticle degradation (17) and from insect midgut (5,8,24) where they are involved with digestion. This report describes, for the first time, an aminopeptidase isolated from hemolymph plasma of an insect, the gypsy moth *Lymantria dispar* (Lepidoptera: Lymantriidae). Some of the biochemical and physiological characteristics of the enzyme are presented along with speculation on its function.

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MATERIALS AND METHODS

Animal Rearing

The culture of *L. dispar* used in this study originated at the USDA/APHIS Methods Development Laboratory [Strain NJ-SS; reference (14)] and is maintained according to the method of Bell *et al.* (3). Details of growth and rearing conditions are in Masler *et al.* (11). Larvae are maintained under a 16-hr light: 8-hr dark photoperiod, 25°C, 50–60% relative humidity. Under these conditions, female larvae go through four molts during a 14- to 16-day period after hatch. Late in the fourth instar, larvae are examined daily between 12 noon and 4 PM (lights on at 6 AM) for evidence of an approaching molt (indicated by head capsule slippage) to

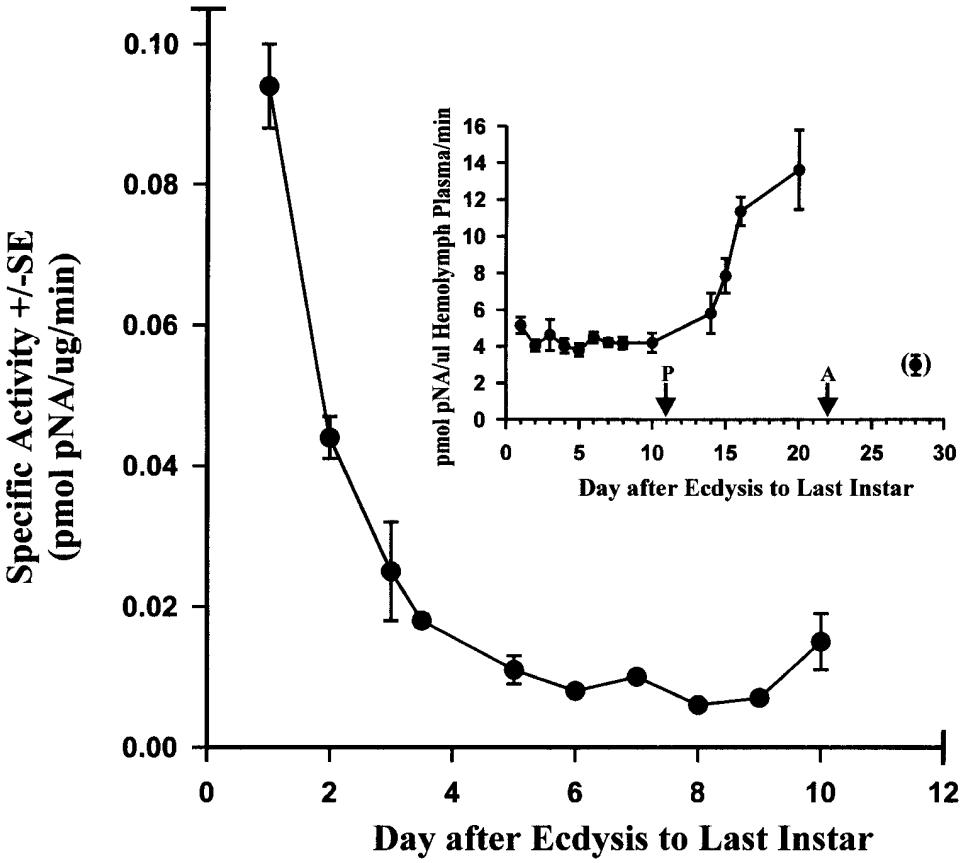

FIG. 1. Changes in total hemolymph plasma aminopeptidase-like activity during the last larval instar and pupal stage, and specific activities during the last larval instar, of female *Lymantria dispar*. *L. dispar* female larvae were sampled starting within 24 hr of molting to the last (fifth) larval instar and at 12- to 24-hr intervals during the instar. For each larva in the sample, total protein (microBCA assay) and total aminopeptidase activity (aminoacyl-4-nitroanilide assay) were determined per μl of plasma. Each data point represents the mean ± SE from 4–14 separate insects. (Inset) Total plasma aminopeptidase activity is expressed as pmol of para-nitroaniline (pNA) produced by the action of 1 μl of hemolymph plasma per minute. A, adult eclosion; P, larval-pupal molt. Data point in parentheses is for 6-day-old adult females. Each data point represents the mean ± SE of 4–13 separate insects.

the last (fifth) larval instar. Each day (12 noon–4 PM), newly molted larvae are collected and designated as day-1, fifth instar. Precision of staging is ±12 hr.

Hemolymph Collection and Enzyme Preparation

Hemolymph was collected from fifth instar female larvae by means of a small puncture with a 26-gauge needle just above the proleg. An abdominal puncture was made in pupae and adults, taking care not to collect fat body tissue. Larvae each yielded up to 100 μl of hemolymph, and pupae or adults yielded 1–5 μl per animal. Collections were made between 9 AM and 12 noon. Although individual insects were not monitored for the developmental profile (Fig. 1, inset), some were bled on more than 1 day but never on consecutive days. Released hemolymph was collected with a polypropylene-tipped pipette and transferred to a polypropylene tube on ice. Pilot experiments revealed that > 95% of total hemolymph aminopeptidase activity detected with our assay was present in the 10,000 *g* supernatant of whole hemolymph. This 10,000 *g* supernatant is referred to as hemolymph plasma. This hemolymph plasma (or plasma) rather than whole hemolymph itself was used for enzyme preparations, activity analyses and purification. Hemolymph used immediately after collection was diluted 1:3 with physiological saline (15), centrifuged 10,000 *g*, 10 min at 4°C and the plasma collected and held on ice. For large-scale preparation (6.5 ml of hemolymph) for enzyme purification, hemolymph was collected into 1.5-ml polypropylene tubes (~ 100 μl per tube), each containing 0.3 ml of 50 mM Tris-HCl, pH 7.6 and a few crystals of phenylthiourea. The hemolymph-filled tubes were centrifuged at 10,000 *g* for 10 min at 4°C and the plasma supernatants collected. Each supernatant was diluted with 10 volumes of cold 50 mM Tris-HCl, pH 7.6. The volume of this mixture was reduced by approximately one half by centrifuging the diluted plasma supernatants at 3,000 *g*, 45 min, 25°C in molecular filtration tubes (Centriplus-30; Amicon, Inc., Beverly, MA, U.S.A.). These units retain molecular weights > 30,000. Retained materials were rinsed off of the filters with 50 mM Tris-HCl, pH 7.6, and the concentrates stored at −10°C in 1-ml aliquots each containing approximately 200 μl of hemolymph plasma.

Enzyme was prepared by ion exchange chromatography using a TSK-DEAE-5PW column (7.5 × 75 mm; Beckman Instruments, Palo Alto, CA, U.S.A.) equipped with a guard column (4 × 35 mm) containing Progel-TSK DEAE-5PW (Supelco, Bellefonte, PA, U.S.A.). Mobile phase was 50 mM Tris-HCl, pH 7.6 with a linear gradient of 0–200 mM NaCl over 90 min at a flow rate of 0.5 ml/min. Absorbance was monitored at 210 nm, and fractions were collected in 1.5- or 4.5-ml polypropylene tubes depending upon the experiment. Plasma aliquots (described above) were thawed and fractionated in batches corresponding to 200 μl of

plasma per batch. Fractions were assayed, and those with activity were frozen (−10°C). Active fractions were pooled and then concentrated using the Centricon-30 system. This concentrated the enzyme and desalted the sample, making it amenable for reapplication to the ion exchange column. When all aliquots had been processed (concentrated, desalted), selected active fractions were rerun using the ion exchange system described above. These concentration and fractionation procedures were repeated three more times until a pure enzyme preparation was obtained. Purity was judged by comparing UV profile with activity and by homogeneity on SDS gels.

Enzyme and Protein Assays

Unfractionated hemolymph plasma or column fractions were diluted into assay buffer (200 mM Tris-HCl, pH 7.3) usually at final dilutions of 1:3–1:4. The substrate used was L-alanine-4-nitroanilide hydrochloride (Fluka Chemical Co., Ronkonkoma, NY, U.S.A.) dissolved in HPLC-grade methanol (Fisher Scientific, Springfield, VA, U.S.A.) at 6.7 nmol/μl. The reaction product, para-nitroaniline (pNA), absorbs at 414 nm. One nmol of pNA absorbs 7.44 milliabsorbance units at 414 nm. This value was used to convert net absorbance into nmoles product. Four μl of the enzyme dilution were combined with 230 μl of assay buffer in wells of a 96-well polystyrene microliter plate (Corning Easy Wash Assay Plate; Corning, Inc., Corning, NY, U.S.A.). The plate was covered and equilibrated for 5–7 min at 32°C. Six μl of substrate were added, and the mixture was equilibrated for an additional 5 min at 32°C. Increase in absorbance at 414 nm over time was measured using a Titertek microplate reader (Flow Laboratories, McClean, VA, U.S.A.). The reaction is linear for at least 1 hr. Total protein was estimated by using the microBCA assay (Pierce Chemical Co., Rockford, IL, U.S.A.). The manufacturer's procedures were followed.

Enzyme Inhibitors

Aminopeptidase inhibitors were dissolved in assay buffer on the day of use, and incubations were prepared as described. Final concentrations of inhibitor are indicated for each experiment. Substrate was added after plate equilibration as described above. Inhibitors tested were actinonin (23), amastatin (1), arphamenine B (21) and leuhistin (2), all from Peninsula Laboratories (Belmont, CA, U.S.A.), and bestatin (22), epiamastatin and epibestatin from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Molecular Weight Estimation

High performance-size exclusion chromatography (HP-SEC) was performed using a TSK-G2000 SW_{XL} column (7.8 × 300 mm; Supelco). Mobile phase was 50 mM sodium phosphate, pH 7.0, in 300 mM NaCl at a flow rate of 0.5 ml/min. Absorbance was monitored at 210 nm, and 250-μl fractions were collected in polypropylene tubes. The column was calibrated with urease (480,000), β-amylase (200,000), bovine serum albumin (66,000), trypsinogen (24,000), cytochrome C (12,300) and aprotinin (6,500), all from Sigma Chemical Co.

For SDS-gel electrophoresis, samples were denatured by heating (boiling water bath) for 5 min in sample buffer (10 mM Tris-HCl, pH 8.0, with 1 mM EDTA, 2.5% SDS and 5% β-mercaptoethanol). Instrumentation used for electrophoresis was the Phast System (Pharmacia, Piscataway, NJ, U.S.A.) following the manufacturer's recommendations for running conditions. The denatured samples (approximately 1 μg/μl) were analyzed on a gradient gel (8–25% acrylamide). The precast gel and buffer supply strips were from Pharmacia. Gels were 0.45 mm thick, with 13-mm stacking and 32-mm separation gels. The gel buffer system was 112 mM Tris-acetate, pH 6.4. Buffer in the denaturing buffer supply strips was 200 mM Tricine, 200 mM Tris and 0.55% SDS, pH 7.5. Conditions for native gel electrophoresis were similar to those for SDS except that buffer strips lacked SDS and the sample buffer was 10 mM Tris-HCl, pH 8.0. Staining was with Phast Gel Blue R (Pharmacia) dissolved to 0.1% in 30% methanol in 10% aqueous acetic acid (3:1:6). Gels were destained in aqueous 30% methanol and 10% acetic acid. Molecular weight markers were from Pharmacia. Those used for SDS-gel calibration were lysozyme (14,300), trypsin inhibitor (21,500), carbonic anhydrase (30,000), ovalbumin (46,000), bovine serum albumin (69,000), phosphorylase B (97,400) and myosin (200,000). Molecular weight markers used for native gel calibration were bovine serum albumin, lactate dehydrogenase (140,000), catalase (232,000), ferritin (440,000) and thyroglobulin (669,000).

RESULTS

Aminopeptidase Activity

Hemolymph plasma aminopeptidase-like activity is detected throughout the last larval instar and increases considerably during the pupal stage (Fig. 1, inset). Activity varies between 2 and 6 pmol pNA produced per minute per microliter plasma during the last larval instar, increasing to just under 12 pmol mid-way through the pupal stage (Fig. 1, inset), and activity continues to increase slightly, to 14 pmol just before adult eclosion (Fig. 1, inset, arrow A). In 6-day-old adults, activity has declined to 2 pmol (Fig. 1, inset). Although highest activities are detected in the pupa, this stage resisted our attempts to obtain hemolymph in enough quantity to allow for extensive biochemical analyses. Consequently, hemolymph plasma collection and enzyme purification efforts focus on larval hemolymph, which is readily collected in sufficient volume. Specific activity during the last larval instar is highest on day 1, declines rapidly by day 3 and remains relatively low throughout the remainder of the instar (Fig. 1). All subsequent work was done with hemolymph collected on day 1.

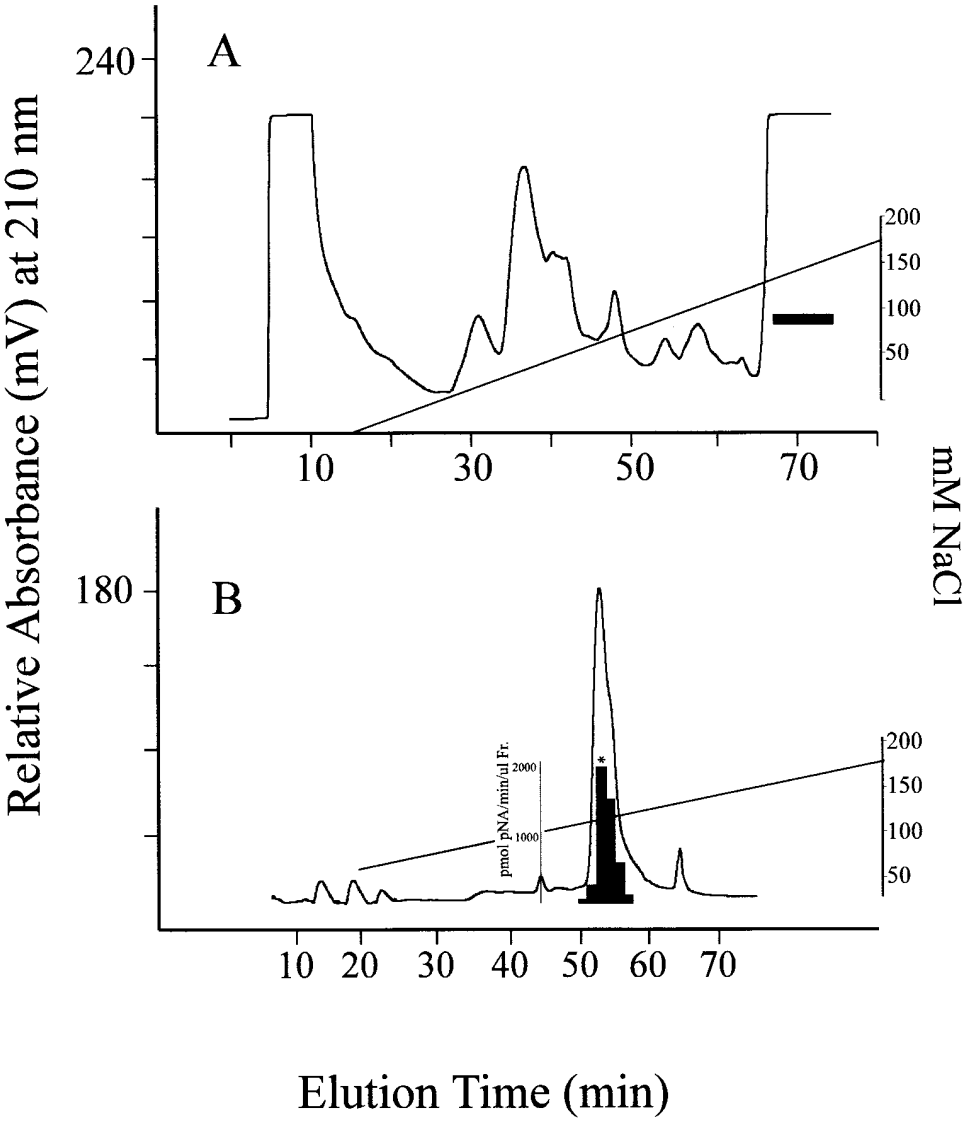

FIG. 2. Ion exchange fractionation of aminopeptidase activity from hemolymph plasma of last instar female *L. dispar* larvae. Plasma (6.5 ml) from 61 larvae was processed as described in the Materials and Methods section. (A) Fractionation of a typical aliquot (450 μl). Fractionation was on a DEAE-5PW column (7.5 × 75 mm) plus guard column (4 × 35 mm) at a flow rate of 0.5 ml/min. A linear gradient from 0–200 mM NaCl was run over 90 min (2.22 mM/min) in 50 mM Tris-HCl, pH 7.6. Fractions were collected each min (0.5 ml/fraction), and 75 μl of each fraction were used for enzyme assay. Fractions with activity are indicated by the horizontal bar. (B) Final ion exchange fractionation. Elution conditions were as described for panel A. Thirty microliters of each fraction were used for the assay. Fractions with enzyme activity are indicated by vertical bars. Activity is expressed as pmol para-nitroaniline (pNA) produced per minute per microliter of fraction. Asterisk indicates fraction that was further processed.

Isolation and Characterization

Hemolymph plasma fractionated by anion exchange chromatography yields aminopeptidase activity eluting in a number of fractions distributed over varying NaCl concentrations (70–80 mM, 120–150 mM, 160–190 mM). However, the majority of activity (70%) is collected in those fractions eluting between 120 and 150 mM NaCl (shaded bar, Fig. 2A). It is these fractions that are processed further. After a series of rechromatographies on the ion exchange system, a homogeneous preparation is obtained (Fig. 2B). Enzyme eluting near 125 mM NaCl (Fig. 2B, asterisk) is collected and designated as AP-125.

An aliquot of AP-125 fractionated on the HP-SEC system yields a single peak of activity eluting at 6–6.5 ml (12–13 min, Fig. 3), corresponding to a molecular weight range of 200–480 kDa (Fig. 3, arrows A and B). AP-125 analyzed by SDS-gel electrophoresis yields a single band migrating near the BSA molecular weight standard of 69,000 (Fig. 4S). A single band migrating near the ferritin molecular weight marker of 440,000 is observed when AP-125 is analyzed on a native gel (Fig. 4N). These data suggest that AP-125 is a hexamer.

Plasma aminopeptidase activity is most susceptible to the inhibitors leuhistin and amastatin (Table 1), inhibiting 90% at 1 and 2 μM, respectively. Bestatin is approximately 10× less potent (90% inhibition at 20 μM). Other inhibitors tested (arphamenine B, actinonin, epiamastatin, epibestatin) are much less effective. Leuhistin and amastatin exhibit different qualitative inhibition characteristics. Leuhistin is a noncompetitive inhibitor of plasma aminopeptidase, and amastatin is competitive (Fig. 5). The IC_{50} for amastatin is three times higher than that for leuhistin (Ta-

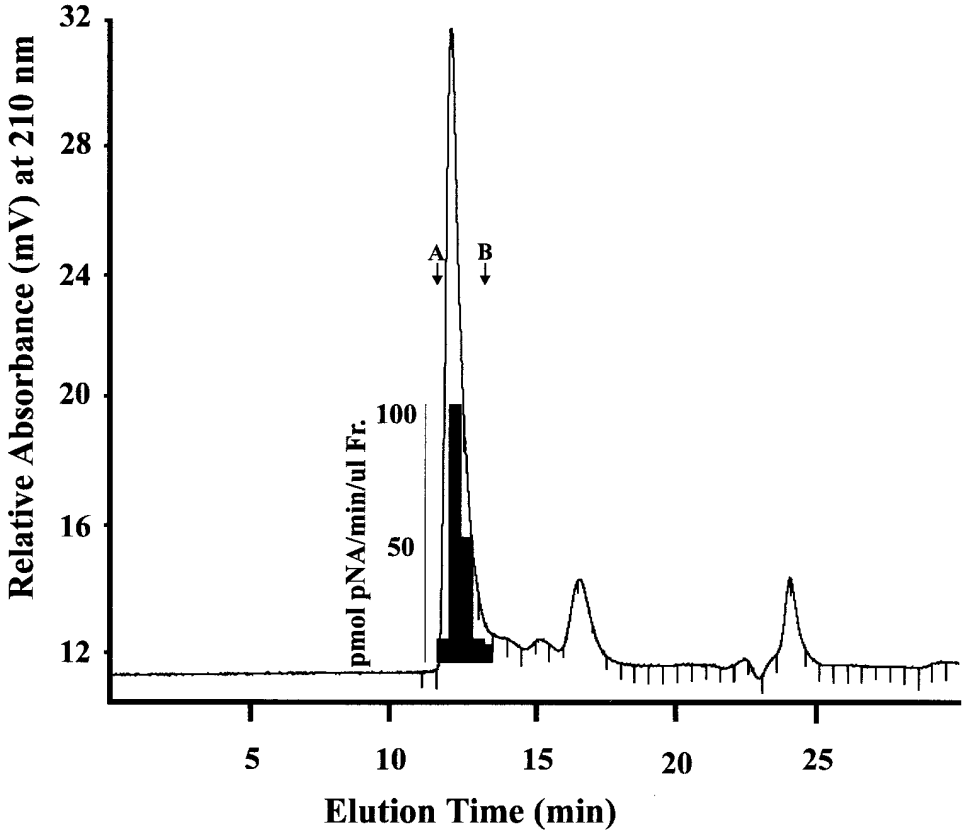

FIG. 3. Purification of aminopeptidase by HP-SEC. An aliquot of the active (54 min) fraction from the final ion exchange step (Fig. 2B, asterisk) was applied to the HP-SEC system. Flow rate was 0.5 ml/min, and fractions were collected from 5–16 min in 0.5-min intervals (250 μl/fraction). Fifty microliters of each fraction were assayed. Activity (shaded area) is expressed as pmol para-nitroaniline (pNA) produced per minute per microliter of fraction. Bracketing molecular weight markers (arrows) used were urease (A, 480,000) and β-amylase (B, 200,000).

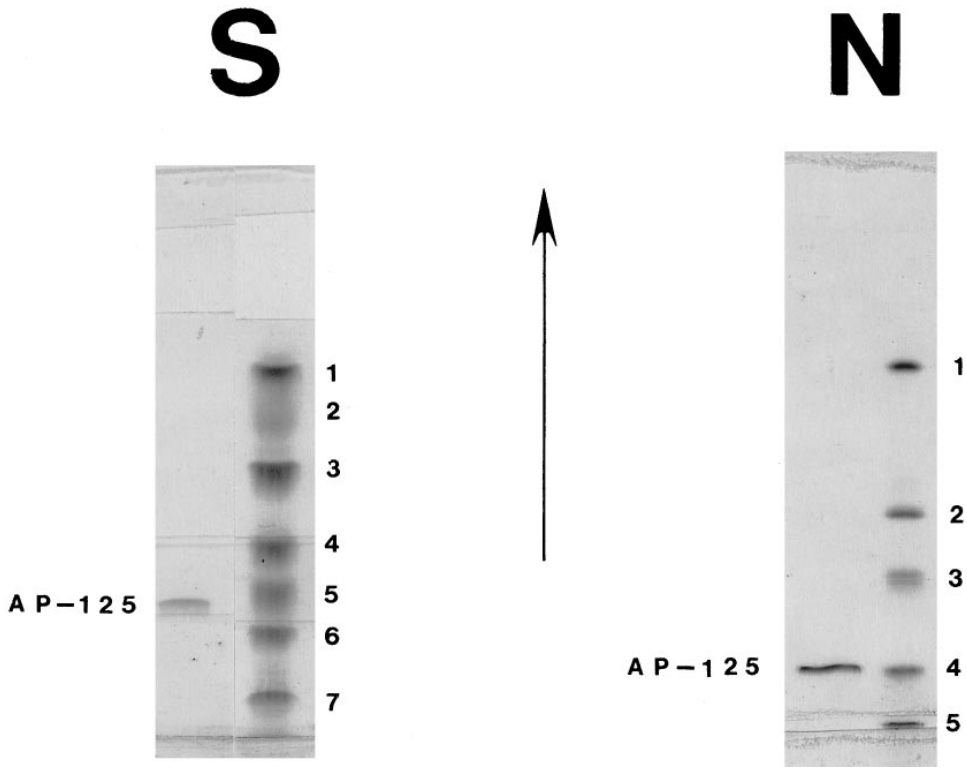

FIG. 4. Polyacrylamide gel electrophoresis analyses of the major aminopeptidase peak from HP-SEC fractionation. (S) An aliquot of the major active fraction of AP-125 eluting at 12.5 min (Fig. 3) was applied to an SDS-PAGE gel system. Molecular weight markers (right lane, section S) were: 1—lysozyme (14,300), 2—trypsin inhibitor (21,500), 3—carbonic anhydrase (30,000), 4—ovalbumin (46,000), 5—bovine serum albumin (69,000), 6—phosphorylase B (97,400), 7—myosin (200,000). (N) An aliquot of the same active fraction used in S was applied to a native PAGE gel system. Molecular weight markers (right lane, section N) were: 1—bovine serum albumin (69,000), 2—lactate dehydrogenase (140,000), 3—catalase (232,000), 4—ferritin (440,000), 5—thyroglobulin (669,000). Arrow indicates direction of current flow.

TABLE 1. Effects of various inhibitors upon the activity of *L. dispar* hemolymph plasma aminopeptidase-like activity

Inhibitors	Concentration tested	Percent inhibition*
Actinonin	100 μM	38
Amastatin	2 μM	88
Amastatin	8 μM	90
Arphamenine B	100 μM	<1
Bestatin	20 μM	90
Epiamastatin	80 μM	20
Epibestatin	80 μM	40
Leuhistin	1 μM	90
Leuhistin	10 μM	95

*Maximum percent inhibition observed when inhibitor is tested in a concentration series from 0.01 to 100 μM.

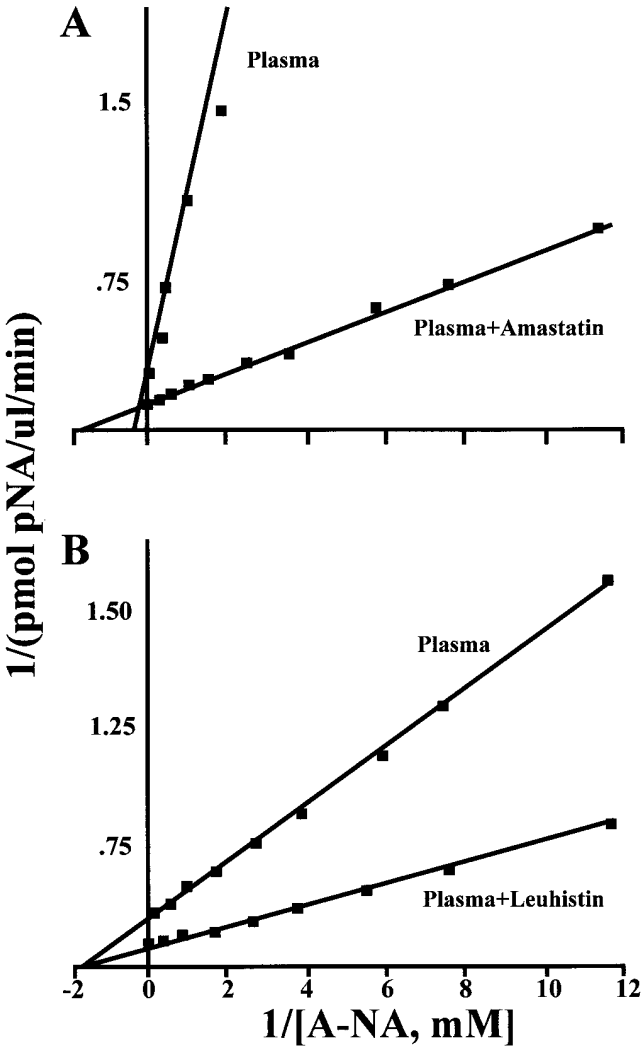

FIG. 5. Inhibition characteristics of amastatin and leuhistin. Lineweaver-Burk plots were generated for 0.1 μM amastatin (A) and 0.01 μM leuhistin (B) at varying concentrations of substrate (L-alanine-4-nitroanilide hydrochloride, A-NA) vs enzymatic activity in hemolymph plasma. pNA, para-nitroanilide.

TABLE 2. K_m and IC_{50} values for *L. dispar* aminopeptidase-like activity in hemolymph plasma and purified AP-125 enzyme

Enzyme source	K_m	IC_{50} amastatin	IC_{50} leuhistin
Plasma	0.53 ± 0.04 mM	0.32 ± 0.07 μM	0.10 ± 0.02 μM
AP-125	0.30 ± 0.04 mM	2.13 ± 0.31 μM	1.69 ± 0.32 μM

Values given are means ± SE of 3–6 separate determinations.

ble 2). The IC_{50}s for each inhibitor are much higher when tested with pure AP-125 enzyme and quantitatively more similar than with plasma. The K_m of plasma aminopeptidase activity is 0.53 ± 0.04 mM compared with a K_m of 0.30 ± 0.04 mM for pure AP-125 (Table 2).

DISCUSSION

Using the conversion of the synthetic substrate L-alanine-4-nitroanilide hydrochloride into alanine and para-nitroaniline as an assay, aminopeptidase activity is detected in hemolymph plasma of last larval instar, pupa, and in day-6 adults of *L. dispar* (Fig. 1, inset). Last instar larvae prove to be most useful for collecting sufficient volumes of hemolymph for isolation and characterization of enzyme. In final instar larvae, highest specific activities are observed early in the instar (Fig. 1), particularly during the first 24 hr following the molt. It is not clear if this high specific activity is functionally related to the molt, but specific activity declines by day 3 and remains low during the mid-instar (Fig. 1). Specific activity exhibits a small increase toward the end of the fifth larval instar as pupation approaches (Fig. 1). Total plasma aminopeptidase activity increases throughout the pupal stage, reaching levels more than twice those seen in the last larval instar (Fig. 1, inset). A sample of hemolymph plasma from the adult 6 days after eclosion reveals a dramatic drop in activity from pupal levels (Fig. 1, inset). The significance, if any, of this apparent association of enzyme activity with development remains to be determined.

A preponderance of aminopeptidase activity is detected in fractions eluting from the ion exchange column between 120 and 150 mM NaCl. Activity in other locations was not isolated but was regularly observed. In preliminary experiments (data not shown), there was some indication of a differential response of aminopeptidase activities, eluting at different locations along the ion exchange salt gradient, to various aminopeptidase inhibitors. In addition, aminopeptidase activity in nonfractionated plasma is not completely inhibited under any of the inhibitor conditions tested (Table 1). This suggests that *L. dispar* hemolymph may contain more than one type of aminopeptidase. The aggressive degradation of peptide by exposure to hemolymph (18) is most likely effected by multiple exo-proteases such as AP-125. The question of multiple plasma aminopeptidases in *L. dispar*, raised by these observations, is one that deserves further examination.

L. dispar plasma aminopeptidase activity is most susceptible to inhibition by leuhistin (90% inhibition at 1 μM) and amastatin (88% inhibition at 2 μM) (Table 1). Leuhistin is an inhibitor of vertebrate aminopeptidase M (membrane-bound; reference 2). Amastatin is an inhibitor of soluble aminopeptidase A (1,17). Assignment of AP-125 to a particular class of aminopeptidase is complicated, since Masler and Wagner (12) and Masler *et al.* (13) reported that membrane-bound aminopeptidase activity in neural preparations from *L. dispar* is also inhibited by amastatin. Bestatin, an inhibitor of aminopeptidase B (19), is about 10-fold less effective than amastatin (Table 1). The optical epi-isomers of amastatin and bestatin (Table 1) are less effective than their enantiomers. Actinonin, an inhibitor of aminopeptidase M (23), and arphamenine B, an inhibitor of aminopeptidase B (21), are essentially ineffective at inhibiting AP-125. The K_ms for hemolymph plasma aminopeptidase and purified AP-125 are 0.53 and 0.30 mM, respectively. These values suggest that the majority of detected activity is present as AP-125. For comparison, the K_m of a membrane-bound aminopeptidase isolated from midgut cell plasma membranes of *Rhynchosciara americana* (Diptera: Sciaridae) range from 0.23–1.5 mM and are dependent upon the aminoacyl napthylamide substrate used (5). The IC_{50}s for hemolymph aminopeptidase activity are 0.32 and 0.10 μM for amastatin and leuhistin, respectively (Table 2). These values reflect the relative potencies of the two inhibitors detected during the inhibitor screening (Table 1). Purified AP-125 shows the same IC_{50} trend. While the quantitative analysis of AP-125 inhibition by amastatin and leuhistin suggests little difference in potency, qualitative analysis suggests that the enzyme is inhibited through different mechanisms by the two inhibitors. Amastatin acts competitively (Fig. 5A), while leuhistin is a noncompetitive inhibitor (Fig. 5B). Amastatin may compete with the substrate for the AP-125 active site, and leuhistin may bind to a noncatalytic site to cause a steric change in the enzyme that affects substrate binding or cleavage. The molecular weight data suggest that AP-125 is a hexamer. It has not been determined if the individual subunits are active or even if they are identical. It is possible that amastatin and leuhistin bind to different subunits.

Aminopeptidases are represented by an extraordinarily broad spectrum of enzymes. Vertebrate enzymes have been characterized from kidney, liver, lens and ascites fluid, among other tissues, and exhibit molecular weights ranging from 53,000–140,000 per subunit and exist as monomers, hexamers and octamers (19). The aminopeptidase isolated from *Manduca sexta* (Lepidoptera: Sphingidae) molting fluid is a hexamer of 240,000 MW (17), and the mid-gut aminopeptidase from *L. dispar* has a molecular weight of 100,000 as determined by SDS-PAGE (24). The molting fluid enzyme is inhibited 86% by 20 μM amastatin, while the midgut enzyme is inhibited 83% by 100 μM bestatin. AP-125 bears little resemblance to any of the insect aminopeptidases reported thus far. This suggests that the variety of insect aminopeptidases will challenge the vertebrates in complexity, with regard to both enzyme structure and tissue location. The precise physiological function of AP-125 has not yet been determined. However, the presence of numerous potential peptide substrates in hemolymph, incomplete inhibition of hemolymph plasma aminopeptidase activity by any single inhibitor and observed changes in activity levels during development suggest that AP-125 may be a member of a family of regulatory hemolymph aminopeptidases.

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